Virgibacillus chiguensis sp. nov., a novel halophilic bacterium isolated from Chigu, a previously commercial saltern located in southern Taiwan

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A Gram-positive, motile, endospore-forming, irregular rod-shaped (0.7–0.9×2.5–5.0 µm), halophilic bacterial strain, NTU-101^T, was isolated from Chigu saltern in southern Taiwan, previously used as a salt production field. The isolate was characterized taxonomically based on biochemical and molecular approaches. It grows optimally at 40 °C and in the presence of 5–10 % NaCl. Strain NTU-101^T has cell-wall peptidoglycan based on *meso*-diaminopimelic acid and MK-7 as the predominant menaquinone. Major polar lipids are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. Anteiso-C_{15:0}, iso-C_{15:0} and anteiso-C_{17:0} are the major fatty acids. The DNA G+C content was 37.3 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed its affiliation to the genus *Virgibacillus*. DNA–DNA relatedness values between strain NTU-101^T and *Virgibacillus dokdonensis* and *Virgibacillus pantothenicus* were 17.5 and 21.5 %, respectively. Based on the phenotypic and phylogenetic properties, strain NTU-101^T (=BCRC 17637^T=CGMCC 1.6496^T) was classified as a novel strain of *Virgibacillus* species, for which the name *Virgibacillus chiguensis* sp. nov. is proposed.

The genus *Virgibacillus* was first proposed by Heyndrickx *et al.* in 1998. At the time of writing, 12 species have been validly described: *Virgibacillus pantothenicus* (Heyndrickx *et al.*, 1998), *Virgibacillus proomii* (Heyndrickx *et al.*, 1999), *Virgibacillus carmonensis*, *Virgibacillus necropolis*, *Virgibacillus picturae* (Heyrman *et al.*, 2003), *Virgibacillus marismortui*, *Virgibacillus salexigens* (Heyrman *et al.*, 2003), *Virgibacillus halodenitrificans* (Yoon *et al.*, 2004), *Virgibacillus dokdonensis* (Yoon *et al.*, 2005), *Virgibacillus koreensis* (Lee *et al.*, 2006), *Virgibacillus olivae* (Quesada *et al.*, 2007) and *Virgibacillus halophilus* (An *et al.*, 2007). In this study, a morphological, biochemical and phylogenetic characterization of a halophilic *Virgibacillus*-like strain, NTU-101^T, was carried out. A single isolate of strain NTU-101^T was obtained from soil sediment collected from Chigu saltern, southern Taiwan. The isolate was cultured using a standard dilution method. Briefly, soil sediment was dissolved in distilled water, diluted 1:1 with solutions of 5, 10, 15 and 20 % (w/v) NaCl and plated onto modified DSM 372 agar plates. The agar plate pH was adjusted to 7.0 with 1 M KOH. The plates were then incubated at 37 °C for 7 days. Single colonies with different morphology were

selected for growth tests. The salt requirement of the isolates was tested using DSM 372 medium [g l⁻¹: yeast extract (Difco), 5; Casamino acids (Difco), 5; MgSO₄·7H₂O, 5; tri-sodium citrate (Sigma), 3; KCl, 2] containing 5 % (w/v) NaCl. Growth was also measured at various temperatures (10–70 °C), pH values (4–10) and salt concentrations (0–35 %). The growth rate was monitored by spectrophotometry at 660 nm. The Gram reaction was tested using a Gram stain kit (Becton Dickinson), according to the manufacturer's instructions. Physiological characterization was carried out by measuring substrate utilization or oxidation reactions on different carbohydrate sources and amino acids using a GN2 MicroPlate Identification Test panel (Biolog). Enzyme activity was determined using the API ZYM system (bioMérieux). Morphology was studied by scanning electron microscopy (SEM). The sample was fixed and processed according to the recommended procedure for specimen preparation (Anton *et al.*, 2002). Gold (approx. thickness 5 nm) was used to coat the samples prior to examination with a scanning electron microscope (Topcon).

Using SEM, strain NTU-101^T was observed to be rod-shaped. Cultural, physiological and biochemical properties are shown in Table 1. Growth tests with different

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NTU-101^T is EF101168.

Table 1. Differential phenotypic characteristics of NTU-101^T and other *Virgibacillus* species

Taxa: 1, NTU-101^T; 2, *V. pantothenicus*; 3, *V. dokdonensis*; 4, *V. proomii*; 5, *V. marismortui*; 6, *V. carmonensis*; 7, *V. necropolis*; 8, *V. picturata*; 9, *V. halodenitrificans* (Yoon *et al.*, 2005); 10, *V. olivae* (Quesada *et al.*, 2007); 11, *V. halophilus* (An *et al.*, 2007). +, Positive; –, negative; w, weakly positive; ND, no data available; v, variable; E, ellipsoidal; S, spherical; C, central; ST, subterminal; T, terminal; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PLs, unidentified phospholipids; APL, unknown aminophospholipid; tr, trace.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Spore shape	S or E	S or E	S or E	S or E	E	E or S	E	E	E	S or E	E
Spore position	T or ST	T or ST	T or ST	T or ST	T or ST	ST	C, ST or T	C, ST or T	T or ST	T or ST	S
Gram stain	+	+	v	+	+	+	+	+	v	+	+
Pigmentation	–	–	–	–	–	Pink	–	–	–	Yellow	Yellow
Anaerobic growth	+	+	+	+	–	–	–	–	+	–	–
Growth at:											
0.5 % NaCl	+	w	+	w	–	–	w	–	v (–)	+	+
25 % NaCl	+	–	–	–	–	–	–	w	v (+)	–	–
pH 10	–	–	ND	–	–	ND	ND	ND	–	–	+
Temperature range (°C)	15–55	15–50	15–50	15–50	15–50	10–40	10–40	15–45	10–45	20–45	5–45
Nitrate reduction	+	v	–	–	+	+	+	–	+	+	+
H ₂ S production	–	v	–	–	+	–	–	+	–	ND	–
Hydrolysis of:											
Aesculin	+	+	+	+	+	w	–	+	v (–)	+	+
Casein	+	+	+	+	+	+	+	+	+	+	ND
Gelatin	+	+	+	v	+	–	w	+	+	+	+
Growth on:											
D-Glucose	+	w	+	w	–	–	+	w	+	–	ND
D-Fructose	+	–	+	–	–	–	–	–	v (+)	+	ND
Sucrose	+	–	+	+	+	+	+	+	+	–	ND
Acid production from:											
D-Galactose	+	–	+	+	–	–	–	w	+	–	w
D-Glucose	+	–	+	+	+	–	w	w	+	–	+
D-Fructose	+	–	+	+	+	–	w	w	+	+	+
D-Mannose	+	–	+	+	+	–	w	w	+	–	+
D-Melibiose	+	–	–	–	–	–	–	–	–	ND	–
D-Rhamnose	–	+	–	v	–	–	–	–	–	–	–
D-Trehalose	–	+	–	+	–	–	w	–	+	ND	+
D-Mannitol	w	–	–	–	–	–	–	–	v (+)	–	+
Major polar lipids	PG, DPG, PE, PLs	PG, DPG, PE, PLs	PG, DPG, PE, PLs	PG, DPG, PE, PLs	PG, DPG, PLs	PG, DPG, PE, PLs	PG, DPG, PLs	PG, DPG, PLs	PG, DPG, PLs	ND	ND
Presence of APL	–	+	–	tr	tr	–	–	–	ND	ND	ND
DNA G + C content (mol%)	37.3	36.9–38.3	36.7	36.8–37.0	39.0–42.8	38.9	37.3	39.5–40.0	38–39	33.4	42.6

concentrations of NaCl indicated that strain NTU-101^T was able to grow in the presence of up to 25 % NaCl, whereas *V. dokdonensis* and *V. pantothenicus* are unable to survive under such conditions. Strain NTU-101^T and *V. dokdonensis* were able to produce acid from D-galactose, D-glucose, D-fructose, D-mannose and D-melibiose, whereas *V. pantothenicus* was not. An unidentified aminophospholipid found in *V. dokdonensis* and *V. pantothenicus* was not found in strain NTU-101^T.

The G + C content of the DNA was determined by the method of Mesbah *et al.* (1989). The nucleotide mixtures were separated by HPLC (JASCO) using a Phenomenex

C18 column. A flow rate of 1.0 ml min^{–1} at a temperature of 37 °C was used and the DNA was quantified by measuring A₂₆₀. Non-methylated bacteriophage lambda DNA (Sigma) was used as the calibration reference. Fatty acid composition was represented by fatty acid methyl esters (FAMES). Cultures were transferred onto Trypticase Soy Agar (TSA) plates containing 3 % Trypticase Soy Broth, 1.5 % Bacto-Agar (Difco) and 7 % NaCl for 24 h at 37 °C. Single colonies were taken with a platinum inoculating loop and transferred to 10 ml Teflon centrifuge tubes equipped with Teflon screw caps (Nalge Nunc International). The extraction of fatty acid was carried out as described by Heyrman *et al.* (1999). FAME profiles

were obtained by GLC using a model 6890N gas chromatograph (HP) as described by Descheemaeker & Swings (1995). The resulting profiles were identified with microbial identification software (MIDI) and a standardized calibration mixture. The isomer of diaminopimelic acid in the peptidoglycan was determined by using the method of Komagata & Suzuki (1987). The identity of the quinines was determined by HPLC (Shin *et al.*, 1996). Analysis of polar lipids by two-dimensional TLC was performed as described by Komagata & Suzuki (1987).

The majority of fatty acids detected in strain NTU-101^T were branched fatty acids (87%); anteiso-C_{15:0} and anteiso-C_{17:0} were present at 52.9 and 15.9%, respectively (Table 2). Other branched fatty acids present were iso-C_{14:0} (1.8%), iso-C_{15:0} (12.1%), iso-C_{16:0} (4.2%) and iso-C_{17:0} (1.5%). Branched fatty acids comprise of 93 and 97.9% of total fatty acids in *V. dokdonensis* and *V. pantothenicus*, respectively. This indicates that strain NTU-101^T differs from *V. dokdonensis* and *V. pantothenicus* with respect to fatty acid composition. The major polar lipids were phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine with some unidentified phospholipids. Strain NTU-101^T has cell-wall peptidoglycan based on meso-diaminopimelic acid, with MK-7 as the predominant menaquinone. This profile is similar to other members of the genus *Virgibacillus*.

Nucleic acids of isolates were extracted using the FastDNA Spin kit (Bio 101), according to the manufacturer's instructions. Two universal primers (9F and 1492R) were used for amplification of the 16S rRNA gene (Stackebrandt & Liesack, 1993). The amplification was performed with the GeneAmp PCR system 9700 (Applied Biosystems). Amplicons were later sequenced (Mission Biotech) and aligned with representatives from genus *Virgibacillus* and related taxa using multiple sequence alignment software (CLUSTAL W 1.82; Thompson *et al.*, 1994). A phylogenetic tree was constructed by the neighbour-joining and UPGMA methods with a bootstrap robustness of 1000 using PHYLIP package 3.6b.

The neighbour-joining tree based on the 16S rRNA gene sequence (1465 nt) showed that strain NTU-101^T is most

closely related to *V. dokdonensis* at a bootstrap level of 99.2%. The remaining *Virgibacillus* species clustered within a clade (Fig. 1). Of particular note is the fact that strain NTU-101^T is topologically distinct from *V. koreensis*, a novel bacterium discovered in a similar salt field environment (Lee *et al.*, 2006). Phylogenetic trees based on maximum-likelihood and maximum-parsimony algorithms were also constructed, and although they demonstrated a slightly different tree topology, the relationships among the various species of *Virgibacillus* remained similar (data not shown). Strain NTU-101^T exhibited 16S rRNA gene sequence similarity of 99.6 and 97.5% to *V. dokdonensis* and *V. pantothenicus*, respectively. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) and values of 17.5 and 21.5% were obtained for strain NTU-101^T with *V. dokdonensis* and *V. pantothenicus*, respectively.

Strain NTU-101^T grew in higher concentrations of NaCl (up to 30%) than any previously described species of *Virgibacillus*. Acid production in strain NTU-101^T also varied compared to other species of *Virgibacillus*. Therefore, differences in terms of biochemical, physiological and phylogenetic relationships are sufficient to suggest that strain NTU-101^T is a novel species of the genus *Virgibacillus*, for which the name *Virgibacillus chiguensis* sp. nov. is proposed.

Description of *Virgibacillus chiguensis* sp. nov.

Virgibacillus chiguensis (chi.gu.en'sis. N.L. masc. adj. *chiguensis* of chigu, a disused salt production field in southern Taiwan from where the strain in this study was isolated).

Cells are rod-shaped, 0.7–0.9 × 2.5–5.0 µm. Gram-positive and motile by means of peritrichous flagella. Terminal or subterminal spore position. Colonies are irregular to regular, flat and translucent, and milky white in colour. After 72 h incubation at 37 °C on DSM 372 agar with 10% (w/v) NaCl, the colony diameter was 5–7 mm. Growth occurs between 0 and 30% NaCl; optimal growth occurs at 5–10% NaCl. Optimal growth occurs at 40 °C; growth was observed at 15–50 °C, but not under 10 °C. The pH range

Table 2. Percentage composition of major fatty acids in *Virgibacillus chiguensis* sp. nov. and related *Virgibacillus* species

Species: 1, *V. chiguensis* sp. nov.; 2, *V. dokdonensis* (Yoon *et al.*, 2005); 3, *V. pantothenicus*; 4, *V. proomii*; 5, *V. marismortui*; 6, *V. carmonensis*; 7, *V. necropolis*; 8, *V. salexigens*; 9, *V. halodenitrificans* (Lee *et al.*, 2006); 10, *V. olivae* (Quesada *et al.*, 2007); 11, *V. halophilus* (An *et al.*, 2007). tr, Trace amount.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
Iso-C _{14:0}	1.8	4.7	2.3	2.4	2.8	3.3	2.2	2.4	7.4	2.0	5.6
Iso-C _{15:0}	12.1	19.4	7.1	15.1	39.0	5.7	3.8	15.1	2.3	33.7	21.2
Anteiso-C _{15:0}	52.9	34.4	55.3	38.4	33.9	58.2	67.8	38.4	51.8	28.4	35.8
Iso-C _{16:0}	4.2	12.3	7.2	7.3	4.3	6.4	4.1	7.3	11.7	3.9	10.3
Iso-C _{17:0}	1.5	7.2	2.0	6.3	5.6	0.9	0.8	6.3	tr	10.1	1.8
Anteiso-C _{17:0}	15.9	15.4	23.9	24.9	8.5	11.1	12.1	24.9	19.5	9.21	16.1

Fig. 1. Neighbour-joining tree showing phylogenetic topology of strain NTU-101^T with other *Virgibacillus* species and related taxa based on the 16S rRNA gene sequence. Bootstrap values from 1000 resamplings are indicated.

for growth is 5–9, with an optimum of 7.5. Growth occurs under aerobic conditions. Nitrate reduction-positive, H₂S production-negative. Aesculin, casein and gelatin are hydrolysed. Starch and Tweens 20 and 80 are hydrolysed. Voges–Proskauer reaction is negative. Indole is not produced. Arginine dihydrolase and lysine decarboxylase are absent. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase and α -mannosidase are present, but leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase and α -fucosidase are absent. Acid is produced from D-cellobiose, D-fructose, D-glucose, D-galactose, D-mannose, D-melibiose, D-ribose, D-sorbitol, sucrose, lactose and maltose. Acid is not produced from L-arabinose, D-melezitose, D-raffinose, D-trehalose and D-xylose. The following substrates are utilized: D-glucose, D-fructose, sucrose, D-cellobiose, D-mannose and maltose. D-Trehalose, benzoate, succinate, L-malate, formate and L-glutamate are not utilized. Major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. The cell-wall peptidoglycan contains meso-diaminopimelic acid, and MK-7 is the predominant menaquinone. The major fatty acids are anteiso-C_{15:0} (51.9%), anteiso-C_{17:0} (15.9%) and iso-C_{15:0} (12.1%). The DNA G + C content is 37.3 mol%.

The type strain, NTU-101^T (=BCRC 17637^T=CGMCC 1.6496^T), was isolated from Chigu, a disused salt field located in southern Taiwan.

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